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Molecular Microbiology (1996) 19(6), 1215–1223

Identification of a second family of high-molecular-weight adhesion proteins expressed by non-typable *Haemophilus influenzae*

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Summary

We previously reported that two surface-exposed high-molecular-weight proteins, HMW1 and HMW2, xpressed by a prototypic strain of non-typable *Haemophilus influenzae* (NTHI), mediate attachment to human epithelial cells. These proteins are members of a family of highly immunogenic proteins common to 70–75% of NTHI strains. NTHI strains that lack HMW1/HMW2-like proteins remain capable of efficient attachment to cultured human epithelial cells, suggesting the existence of additional adhesion molecules. We reasoned that characterization of high-molecular-weight immunogenic proteins from an HMW1/HMW2-deficient strain might identify additional adhesion proteins. A genomic library was prepared in λEMBL3 with chromosomal DNA from non-typable *Haemophilus* strain 11, a strain that lacks HMW1/HMW2-like proteins. The library was screened immunologically with convalescent serum from a child naturally infected with strain 11, and phage clones expressing high-m lecular-weight recombinant proteins were identified by Western blot analysis. One clone was identified that expressed a protein with an apparent molecular mass greater than 200 kDa. Transformation of non-adherent *Escherichia coli* strain DH5α with plasmids containing the genetic locus encoding this pr tein gave rise to *E. coli* transformants that adhered avidly to Chang conjunctival cells. Subcloning and mutagenesis studies localized the DNA conferring the adherence phenotype to a 4.8 kbp fragment, and nucleotide sequence analysis further localized the gene encoding the adhesion protein to a 3.3 kbp open reading frame predicted to encode a protein of 114 kDa. The gene was designated *hia* for *Haemophilus influenzae* adhesin. Southern analysis revealed an *hia* homologue in 13 of 15 HMW1/HMW2-deficient non-typable *H. influenzae* strains. In contrast, the *hia* gene was not present in any of 23 non-typable *H. influenzae* strains which expressed HMW1/HMW2-like proteins. Identification of this second family of high-molecular-weight adhesion proteins suggests the possibility of developing vaccines based upon a combination of HMW1/HMW2-like proteins and Hia-like proteins which would be protective against disease caused by most or all non-typable *H. influenzae*.

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Introduction

Non-typable *Haemophilus influenzae* are non-encapsulated Gram-negative organisms which are common inhabitants of the human upper respiratory tract (Kuklinska and Kilian, 1984). These organisms cause a variety of common mucosal surface infections such as otitis media, sinusitis, conjunctivitis, and chronic bronchitis (Murphy and Apicella, 1987). A critical first step in the pathogenesis of non-typable *Haemophilus* disease involves colonization of the upper-respiratory-tract mucosa. The bacterial molecules that facilitate this process of colonization have yet to be fully characterized. However, in previous work, we identified a family of high-molecular-weight proteins important in attachment of non-typable *H. influenzae* to human epithelial cells *in vitro* (Barenkamp and Leininger, 1992; St. Geme *et al.*, 1993). The two closely related adhesion proteins expressed by our prototype strain were designated HMW1 and HMW2. Approximately 70–75% of epidemiologically unrelated non-typable *Haemophilus* strains express antigenically related HMW1/HMW2-like proteins (Barenkamp and Leininger, 1992). Most of the 25–30% of strains that do not express such proteins also demonstrate high levels of adherence *in vitro* (S. J. Barenkamp and J. W. St. Geme, unpublished), supporting the existence of additional adhesion molecules.

The HMW1/HMW2-like proteins were initially identified and characterized because they were observed to be the immunodominant surface-exposed proteins during the course of natural non-typable *H. influenzae* infecti n

(Barenkamp and Bodor, 1990). When infection is caused by non-typable *Haemophilus* strains that do not express HMW1/HMW2-like proteins, surface-exposed high-molecular-weight proteins are still the immunodominant species noted during infection (Barenkamp and Bodor, 1990; S. J. Barenkamp and J. W. St. Geme, unpublished). We reasoned that characterization of the immunogenic high-molecular-weight proteins expressed by a non-typable *Haemophilus* strain that does not express HMW1/HMW2-like adhesion proteins, yet which does demonstrate good *in vitro* adherence, might allow us to identify additional *Haemophilus* proteins important in bacterial adhesion. In this report, we describe the use of this strategy to identify a novel gene locus encoding another non-typable *Haemophilus* high-molecular-weight adhesion protein.

Results

Selection of the prototype HMW1/HMW2-deficient strain

The HMW1/HMW2-deficient non-typable *H. influenzae* strain selected for study was strain 11. This strain was originally isolated from the middle-ear fluid of a child with acute otitis media. When examined using a whole-cell radioimmunoprecipitation assay, convalescent serum from the child naturally infected with this strain contained antibodies in high titre directed predominantly against surface-exposed high-molecular-weight proteins with molecular masses greater than 100 kDa (Barenkamp and Bodor, 1990). Furthermore, examination of strain 11 in our standard *in vitro* adherence assay demonstrated a high level of attachment to Chang conjunctival cells. Based on these observations, we reasoned that strain 11 might possess a novel high-molecular-weight adhesion protein, distinct from HMW1 and HMW2.

Isolation and characterization of a recombinant phage and plasmid subclones expressing the strain-11 high-molecular-weight adhesion protein

A strain 11 λEMBL3 library was screened immunologically with the convalescent serum described above. Immunoreactive clones were examined by Western blotting for expression of high-molecular-weight proteins with apparent molecular masses greater than 100 kDa. A single phage clone was recovered which expressed the high-molecular-weight protein that is the focus of this study. This clone, designated 11-17, expressed a protein with an apparent molecular mass of greater than 200 kDa (Fig. 1). A partial restriction map of this phage clone is shown in Fig. 2.

To test the hypothesis that the recombinant protein

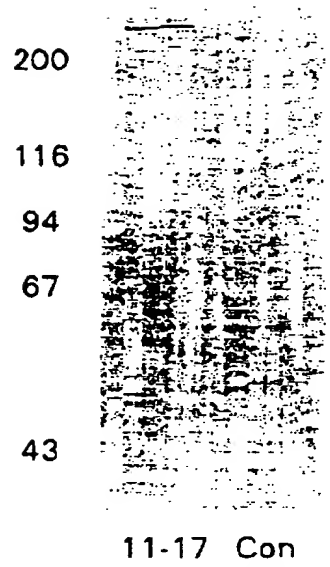

Fig. 1. Western immunoblot assay with a phage lysate expressing the high-molecular-weight adhesion protein encoded by the *hia* gene and a control lysate from an unrelated recombinant phage. Lysates were probed with the *E. coli*-adsorbed convalescent serum sample of a child naturally infected with non-typable *Haemophilus* strain 11. This serum sample demonstrated high-titre antibody against strain 11 high-molecular-weight proteins in a whole-cell radioimmunoprecipitation assay (Barenkamp and Bodor, 1990).

expressed by phage 11-17 was important in adhesion, we constructed two plasmid subclones designated pHMW8-3 and pHMW8-4 (Fig. 2). These plasmids were introduced into the non-adherent *Escherichia coli* strain DH5α, and the resultant strains were examined for adherence to Chang cells. As shown in Table 1 and Fig. 3, both recombinant strains were capable of efficient attachment, similar in magnitude to that demonstrated by DH5α expressing the previously described HMW1 protein. To further localize the segment of DNA involved in *in vitro* attachment, additional subclones designated pHMW8-5 and pHMW8-7 were constructed (Fig. 2). Each of these plasmids also conferred a capacity for attachment (Table 1), suggesting that a 4.8 kbp *Xba*I–*Nru*I fragment contains sufficient genetic material for the adherent phenotype. To confirm this conclusion, we constructed the plasmid pHMW8-6, a plasmid in which a kanamycin cassette was inserted into pHMW8-4 at a unique *Spe*I site between the *Xba*I and *Nru*I sites. As predicted, DH5α harbouring pHMW8-6 demonstrated negligible attachment, adhering no better than DH5α transformed with the cloning vector alone (Table 1).

Identification of plasmid-encoded polypeptides

In order to identify the plasmid-encoded proteins involved

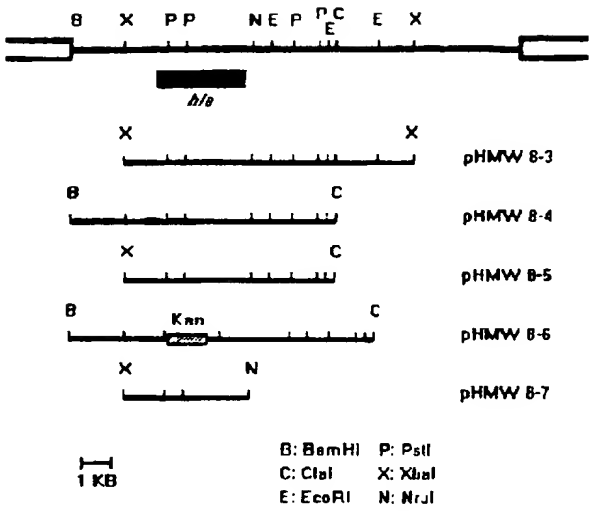

Fig. 2. Partial restriction maps of recombinant λEMBL3 phage clone 11-17 and of pT7-7 plasmid subclones. The solid bar beneath the phage 11-17 restriction map indicates the location of the *hia* gene. Each of the indicated plasmids contains the intact *hia* gene locus, with the exception of pHMW 8-6 in which the *hia* gene is interrupted by a kanamycin-resistance cassette inserted at a unique *Spe*I site. The methods used for construction of each of the plasmids are described in the *Experimental procecures*.

In *in vitro* attachment, we introduced pHMW8-4, pHMW8-5, pHMW8-7, and pHMW8-6 into *E. coli* BL21(DE3). As a negative control, pT7-7 was also transformed into BL21(DE3). In the resulting strains, the T7 promoter was activated with IPTG (final concentration 1 mM), and induced proteins were detected using [^{35}S]-methionine. As shown in Fig. 4, transformants harbouring plasmids pHMW8-4, pHMW8-5, and pHMW8-7 expressed a new protein approximately 116 kDa in size. In addition, we observed a series of proteins that migrated at greater than 200 kDa, corresponding in size to the immunoreactive protein noted in lysates from phage 11-17. BL21(DE3) harbouring pHMW8-6, the pHMW8-4 derivative which contains a kanamycin cassette at the *Spe*I site, failed to express either the 116 kD protein or the larger protein species. Similarly, BL21(DE3) carrying pT7-7 also lacked these proteins.

Table 1. Adherence by *E. coli* DH5α harbouring *hia*- or *hmw1*-containing plasmids.

Strain	% Adherence[a]
DH5α(pT7-7)	0.6 ± 0.2
DH5α(pHMW 8-3)	38.0 ± 3.8
DH5α(pHMW 8-4)	43.3 ± 5.0
DH5α(pHMW 8-5)	41.3 ± 1.3
DH5α(pHMW 8-7)	31.0 ± 3.3
DH5α(pHMW 8-6)	0.6 ± 0.3
DH5α(pHMW1-14)	57.9 ± 5.3

a. Adherence was measured in a 30 min assay and was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. Values are the mean ± SEM of measurements made in triplicate from a representative experiment.

Sequence analysis of the strain-11 adhesion locus

Based on our subcloning and mutagenesis results, we began sequencing the 4.8 kb *Xba*I–*Nru*I fragment present in pHMW8-7. Using this approach, we identified a 3294 bp open reading frame (ORF), which we have designated *hia*, for *Haemophilus influenzae* adhesin. A putative ribosomal binding site with the sequence AAGGTA begins 13 bp upstream of the putative initiation codon. Multiple termination codons are present in all three reading frames both upstream and downstream of the *hia* ORF. The 5′-flanking region of the gene is notable for the presence of 40 consecutive thymines which span the region from 160 to 120 bp upstream of the start codon. The *hia* gene encodes a 1098 amino acid polypeptide, which we have designated Hia, with a calculated molecular mass of 114.1 kDa. This calculated molecular mass is in good agreement with the 116 kDa protein noted in BL21(DE3) derivatives carrying pHMW8-4, pHMW8-5, or pHMW8-7. We speculate that the much larger immunoreactive protein expressed by phage 11-17 is being detected as a multimer in Western blots.

Analysis of sequence upstream and downstream of the *hia* gene revealed additional ORFs in both locations. Upstream is an ORF that begins some distance from the 5′-end of the *Xba*I site from which we began sequencing, and terminates 400 bp from the 5′-end of the *hia* start codon. Beginning approximately 240 bp downstream from the *hia* termination codon is the 5′-end of another ORF, which extends beyond the *Nru*I site.

Protein-sequence-similarity search

A protein-sequence-similarity search was performed with the derived amino acid sequence of the *hia* gene using the Lipman–Pearson TFASTA algorithm and the GenBank/EMBL Nucleotide Sequence Data Library (Devereux *et al.*, 1984). The only proteins in the database that demonstrated significant sequence similarity with the *hia* gene product were two other bacterial proteins: HMW1, the protein we previously identified as being an important adhesin of non-typable *H. influenzae* (St. Geme *et al.*, 1993), and AIDA-I, an adhesion protein expressed by enteropathogenic *E. coli* (Benz and Schmidt, 1992a,b). The initial and optimized scores for the Hia protein–HMW1 sequence comparison were 65 and 146, respectively, with a 387 amino acid overlap. The initial and optimized scores for the Hia protein–AIDA-I sequence comparison were 101 and 170, respectively, with an 833

Fig. 3. Light micrographs of *E. coli* DH5α-derivatives incubated with Chang epithelial cells. Bacteria were incubated with an epithelial monolayer for 30 min before rinsing and staining with Giemsa stain.
A. DH5α carrying pT7-7 (cloning vector alone).
B. DH5α carrying pHMW 8-3.
C. DH5α carrying pHMW 8-4.
The bar represents 2.5 μm.

amino acid overlap. The magnitude of the initial and optimized TFASTA scores and the very large regions of overlap suggest that biologically significant relationships exist between the Hia protein and the two other bacterial adhesins (Lipman and Pearson, 1985).

When the Hia amino acid sequence was compared directly with the sequences of the HMW1 and AIDA-I proteins using the GAP program (Devereux *et al.*, 1984), no long stretches of amino acid identity could be demonstrated. However, 10 of the first 25 amino acids at the N-termini of the Hia protein and HMW1 were identical, and the only cysteine present in the Hia protein was residue 29, a position nearly identical to the position of one of only three cysteine residues present in the HMW1 amino acid sequence. Neither the HMW1 nor the Hia protein has a typical prokaryotic signal sequence. A very long segment is removed from the amino terminus of the HMW1 precursor protein to generate the mature protein (Barenkamp and Leininger, 1992). Whether similar processing occurs in the case of the Hia protein is unclear at present, as we have no information on the amino acid sequence of the N-terminal of the mature Hia protein.

A sequence-similarity search with the open reading frame upstream of the *hia* gene revealed significant homology with the *E. coli* exoribonuclease II protein.

Presence of the hia *locus in unrelated non-typable* H. influenzae *strains*

To define the extent to which the *hia* locus is shared by other non-typable *H. influenzae* strains, we examined a panel of epidemiologically unrelated non-typable *Haemophilus* isolates by Southern hybridization. Chromosomal DNA was digested with *Eco*RI and then probed with a 1.7 kb *Spe*I–*Bst*EII DNA fragment internal to the *hia* gene. Figure 5 depicts the results of a representative Southern blot. Chromosomal DNA from prototype strain 11 is shown in lane 1 and demonstrates a band of hybridization of approximately 10 kbp. Five additional non-typable *Haemophilus* strains which do not express HMW1/HMW2-related proteins are shown in lanes 2–6. Four of these five strains also demonstrate a band of hybridization with the *hia* gene probe. Three non-typable *Haemophilus* strains which do express HMW1/HMW2-related proteins are shown in lanes 7–9. None of these strains demonstrate a band of hybridization.

Of 15 non-typable *H. influenzae* strains deficient in an HMW1/HMW2-related protein, 13 demonstrated evidence of an *hia* homologue. In contrast, of 23 strains that expressed HMW1/HMW2-related proteins, none was found to hybridize with the *hia*-specific probe. Thus, the *hia* gene appears to be restricted to strains that lack HMW1/HMW2-related proteins and is present in most of these HMW1/HMW2-deficient strains.

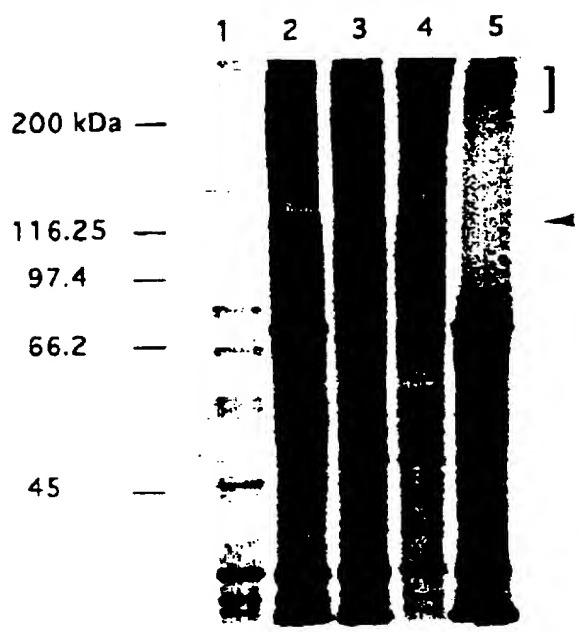

Fig. 4. Autoradiograph of whole cell lysates prepared from *E. coli* strain BL21(DE) transformed with pT7-7-derived plasmids containing the *hia* gene locus. The cellular proteins were separated on 7.5% acrylamide gels prior to autoradiography. The indicated lanes contain the following lysate preparations: lane 1, BL21(DE3)/pT7-7; lane 2, BL21(DE3)/pHMW8-4; lane 3, BL21(DE3)/pHMW8-5; lane 4, BL21(DE3)/pHMW8-7; and lane 5, BL21(DE3)/pHMW8-6. An arrow indicates the overexpressed protein corresponding to the predicted *hia* gene product. A bracket denotes proteins which presumably represent Hia multimers.

Insertional inactivation of the hia *gene in* H. influenzae

To confirm a role for the *hia* gene in attachment by strain 11, we constructed an Hia isogenic mutant strain. To do this, plasmid pHMW8-6 was linearized by digestion with *Nde*I and was transformed into strain 11, followed by selection for kanamycin-resistant colonies. Western immunoblot analysis demonstrated loss of expression of 116 kDa and 250 kDa high-molecular-weight bands in the kanamycin-resistant mutant strain (Fig. 6, compare lanes 1 and 2). Southern analysis confirmed insertion into the *hia* gene (data not shown). The resulting strain 11 *hia*$^-$ mutant was examined for attachment to Chang epithelial cells. As shown in Table 2, attachment by the mutant was reduced roughly 25-fold compared with the parent strain 11.

To provide additional evidence that the *hia* gene encodes a product involved in *in vitro* attachment, we employed pHMW8-6 to insertionally inactivate the *hia* locus of non-typable *Haemophilus* strain 1512. Again, Western immunoblot demonstrated loss of expression of the Hia-like protein and Southern analysis confirmed the allelic exchange (data not shown). Similar to the situation with strain 11, the strain 1512 *hia*$^-$ mutant demonstrated minimal attachment compared with the strain-1512 parent (Table 2).

Discussion

Non-typable *H. influenzae* are human-specific pathogens that normally reside in the upper respiratory tract (Kuklinska and Kilian, 1984). The ability to adhere to the human respiratory epithelium is a crucial factor in promoting persistence of non-typable *H. influenzae* in the human host. Several distinct surface molecules and organelles have been identified that contribute to the *in vitro* adherence of these organisms (Gilsdorf *et al.*, 1992; Noel *et al.*, 1994; St. Geme *et al.*, 1991; 1993; 1994; St. Geme and Cutter, 1995; Sirakova *et al.*, 1994). These same molecules are likely to be important contributors to the organism's survival *in vivo*. The best characterized of the surface-adhesion molecules of non-typable *H. influenzae* are the haemagglutinating pili (Gilsdorf *et al.*, 1992). These organelles, which are helical structures approximately 5 nm in diameter and up to 450 nm in length, promote agglutination of human erythrocytes and attachment to human oropharyngeal epithelial cells (Gilsdorf *et al.*, 1992).

Studies from a number of investigators have suggested the existence of adhesins in non-typable *H. influenzae* distinct from the haemagglutinating pili. St. Geme and coworkers recently isolated and characterized a gene designated *hap* which encodes an IgA protease-like protein that promotes attachment and low-level invasion in experiments with Chang conjunctival cells (St. Geme *et al.*, 1994). In our earlier work, we described and characterized the HMW1/HMW2 family of high-molecular-weight adhesion proteins which appear to be critical to the attachment of non-typable *H. influenzae* to several types of human epithelial and phagocytic cells *in vitro* (Barenkamp and Leininger, 1992; St. Geme *et al.*, 1993; Barenkamp and St. Geme, 1994). Other workers have described surface 'fimbriae', fine filamentous surface structures distinct from the larger 'pili', which are also capable of mediating attachment to human oropharyngeal epithelial cells (Sirakova *et al.*, 1994). All of these previously described adhesion loci are distinct from the *hia* gene locus we describe in this report. The adhesion properties of the various proteins just described have been defined almost exclusively in *in vitro* systems. The contribution of the Hia protein and the other adhesion proteins to the natural colonization process in the human host have yet to be fully characterized.

The *hia* gene locus is predicted to encode a 114 kDa protein. The derived amino acid sequence of the protein demonstrates low-level sequence similarity with two previously described bacterial adhesins: the HMW1 adhesion protein of non-typable *H. influenzae* (Barenkamp

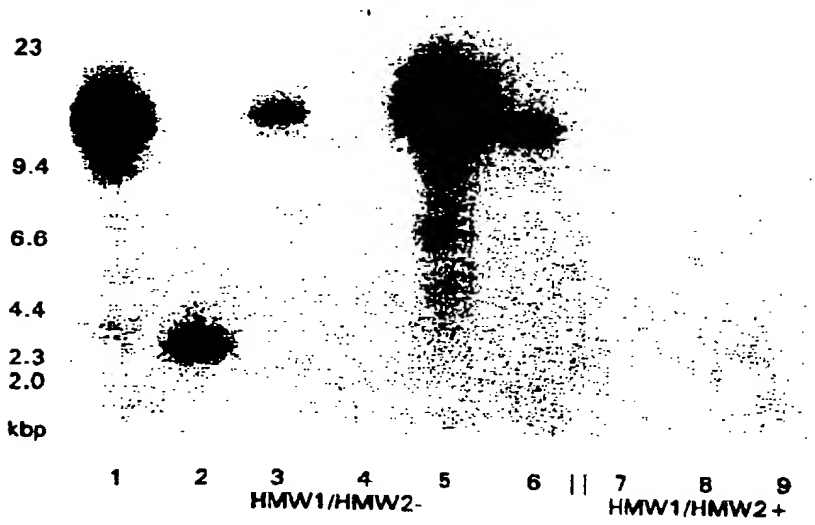

Fig. 5. Southern hybridization analysis of chromosomal DNA from a panel of non-typable *H. influenzae*. Chromosomal DNA preparations from each strain were digested to completion with *Eco*RI, electrophoresed on a 0.7% agarose gel, transferred to a Hybond-N$^+$ membrane and probed with a ^{32}P-labelled *Spe*I–*Bst*EII internal probe from the *hia* gene. Lanes 1–6 contain DNA from strains that do not express HMW1/HMW2-like proteins; lanes 7–9 contain DNA from strains that do express HMW1/HMW2-like proteins.

and Leininger, 1992), and the AIDA-I adhesin of *E. coli* (Benz and Schmidt, 1992a,b). *E. coli* cells which express this latter protein demonstrate a 'diffuse adherence' phenotype, whereby the bacteria adhere evenly to the whole cell surface of HEp-2 and HeLa cells *in vitro* (Benz and Schmidt, 1992b). Comparison of the three sequences does not reveal any extended regions of amino acid sequence identity that might allow identification of common binding domains. Both the HMW1 protein and the AIDA-I adhesin are encoded by gene clusters comprising a structural protein gene and additional accessory genes required for correct processing and surface localization of the actual adhesion proteins (Benz and Schmidt, 1992a,b; Barenkamp and St. Geme, 1994). Interestingly, based upon the adhesion data with *E. coli* transformants expressing the Hia protein, it would appear that the Hia protein is capable of reaching the bacterial surface and mediating *in vitro* adherence, independent of other *Haemophilus* gene products. Alternatively, it is conceivable that *E. coli*-encoded proteins may aid in surface localization of the Hia protein.

The complete genome sequence of *H. influenzae* Rd was recently reported (Fleischmann *et al.*, 1995). An ORF demonstrating sequence similarity to the AIDA-I adhesin of *E. coli* was identified in the Rd sequence. Comparison of this *H. influenzae* Rd ORF with the *hia* gene demonstrates that the two sequences are closely related. The Rd ORF is 894 nucleotides in length and is predicted to encode a protein of 298 amino acids. At the nucleotide level, the Rd ORF is 66% identical with the corresponding *hia* nucleotide sequence and the derived amino acid sequences are 57% identical. The Rd ORF is approximately one-quarter of the size of the *hia* gene, with the size difference explained by the presence of a frameshift 'mutation' in the Rd sequence, which results in the appearance of a premature stop codon. At present, no functional role has been determined for the protein encoded by the Rd ORF.

In earlier studies we reported that 70–75% of non-typable *H. influenzae* expressed the HMW1/HMW2-like high-molecular-weight adhesion proteins. In this study, we examined a panel of non-typable *Haemophilus* strains for the presence of the *hia* gene locus by Southern hybridization, and only a subset of strains demonstrated bands of hybridization: specifically, only those strains that did not express HMW1/HMW2-related proteins. Furthermore, almost all such 'non-expressing' strains that we examined demonstrated bands of hybridization with the *hia* gene probe. This result is consistent with a model in which non-typable *H. influenzae* express either the HMW1/HMW2-like or the Hia protein-like high-molecular-weight adhesion proteins but not both. It is interesting to speculate that there are two groups of strains which represent distinct evolutionary lineages.

In summary, we have cloned and characterized a gene locus, designated *hia*, from a prototype non-typable *H. influenzae* strain that encodes a novel high-molecular-weight adhesion protein. The protein encoded by this locus was originally detected based upon its highly immunogenic character during the course of infection in a child with acute non-typable *H. influenzae* otitis media. Although this protein is expressed by only a subset of non-typable *Haemophilus* strains, its immunogenicity and role as an adhesion protein suggests its potential role as a vaccine candidate. If combined with representative HMW1/HMW2-like proteins, proteins which are major non-pilus adhesins for non-typable *Haemophilus* strains that do not contain an *hia* gene, a vaccine formulation

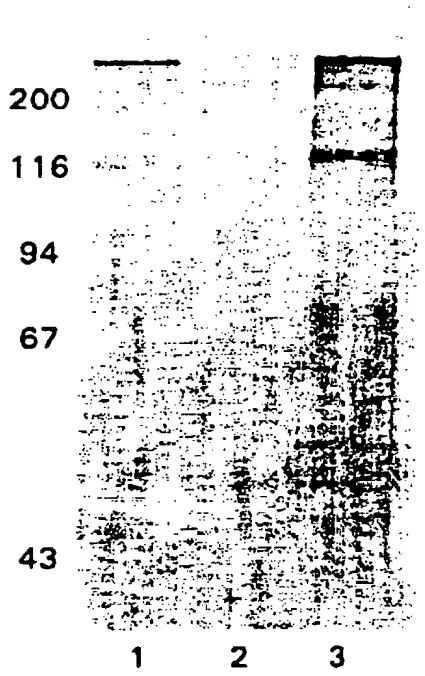

Fig. 6. Western immunoblot assay with cell sonicates from non-typable *H. influenzae* strain 11 (lane 1), and a kanamycin-resistant derivative with an interrupted *hia* gene (lane 2), and with clone 11-17 phage lysate (lane 3). The preparations were probed with an *E. coli*-adsorbed rabbit immune serum raised against a recombinant Hia fusion protein. Size markers are in kDa.

could be envisioned that would be protective against most or all non-typable *H. influenzae*.

Experimental procedures

Bacterial strains, plasmids, and phages

Non-typable *H. influenzae* strain 11 was chosen as a prototypic HMW1/HMW2-deficient strain (Barenkamp and Bodor, 1990; Barenkamp and Leininger, 1992). This strain was isolated in pure culture from the middle-ear fluid of a child with acute otitis media. It was identified as *H. influenzae* by standard methods (Kilian, 1985) and was classified as non-typable based on its failure t agglutinat with a panel of typing antisera for *H. influenzae* types a–f (Burroughs Wellcom Co.) or to show lines of precipitation with these antisera in counter-immunoelectrophoresis assays (Wallace *et al.*, 1981). Additional non-typable *H. influenzae* strains were described previously (Barenkamp and Leininger, 1992).

E. coli strain DH5α was obtained from Gibco BRL. *E. coli* BL21(DE3) was a gift from F. William Studier. Strain BL21(DE3) contains a single copy of the T7 RNA polymerase gene under the control of the *lac* regulatory system (Studier and Moffatt, 1986). M13mp18 and M13mp19 were obtained from New England BioLabs Inc. pT7-7 was the kind gift of Stanley Tabor (Tabor and Richardson, 1985). This vector contains the T7 RNA polymerase promoter ϕ10, a ribosome-binding site, and the translational start site for the T7 gene 10 protein upstream from a multiple cloning site. pUC4K was obtained from Pharmacia and served as the source of the kanamycin-resistance gene.

Table 2. Adherence by non-typable *H. influenzae* strains and isogenic *hia* mutants.

Strain	% Adherence[a]
H. influenzae 11	45.6 ± 4.1
H. influenzae 11/*hia*⁻	1.8 ± 0.7
H. influenzae 1512	68.5 ± 9.3
H. influenzae 1512/*hia*⁻	0.7 ± 0.1

a. Adherence was measured in a 30 min assay and was calculated by dividing the number of adherent bacteria by the number of inoculated bacteria. Values are the mean ± SEM of measurements made in triplicate from a representative experiment.

Culture conditions

H. influenzae strains were grown on chocolate agar supplemented with 1% Isovitale X, on brain–heart infusion agar supplemented with haemin and NAD (BHI–DB agar), or in brain–heart infusion broth supplemented with haemin and NAD (BHIs) (Anderson *et al.*, 1972). These strains were stored at −80°C in brain–heart infusion broth with 25% glycerol, or in skim milk. *E. coli* strains were grown on Luria–Bertani (LB) agar or in LB broth and were stored at −80°C in LB broth with 50% glycerol. For *H. influenzae*, kanamycin was used at a concentration of 20 μg ml^{-1}. Antibiotic concentrations for *E. coli* included the following: ampicillin or carbenicillin at 100 μg ml^{-1}, and kanamycin at 50 μg ml^{-1}.

Molecular cloning and plasmid subcloning

The recombinant phage containing the *hia* gene was isolated and characterized using methods similar to those described previously (Barenkamp and Leininger, 1992). In brief, chromosomal DNA from strain 11 was prepared by a modification of the method of Marmur (1961). *Sau* 3A partial restriction digests of the DNA were prepared and fractionated on 0.7% agarose gels. Fractions containing DNA fragments in the 9–20 kbp range were pooled, and a library was prepared by ligation into λEMBL3 arms. Ligation mixtures were packaged *in vitro* with Gigapack® (Stratagene) and plate-amplified in a P2 lysogen of *E. coli* LE392. Lambda plaque immunological screening was performed as described by Maniatis *et al.* (1982). For plasmid subcloning studies, DNA from recombinant phage was subcloned into the T7 expression plasmid pT7-7 (Tabor and Richardson, 1985). Standard methods were used for manipulation of cloned DNA as described (Maniatis *et al.*, 1982; Silhavy *et al.*, 1984).

The plasmid subclones containing the *hia* gene locus described in this work were constructed as follows. Plasmid pHMW 8-3 was generated by isolating an 11 kbp *Xba*I fragment using DNA purified from recombinant phage clone 11-17 and ligating into *Xba*I-digested pT7-7. Plasmid pHMW8-4 was generated by isolating a 10 kbp *Bam*HI–*Cla*I fragment from clone 11-17 DNA and ligating into *Bam*HI–*Cla*I-digested pT7-7. Plasmid pHMW8-5 was generated by digesting

pHMW8-4 with *Xba*I, isolating the 10.5 kbp fragment and religating. (An additional *Xba*I restriction site not shown in Fig. 2 is present adjacent to the *Bam*HI site of pHMW8-4; this site is derived from the polylinker region of pT7-7.) Plasmid pHMW8-6 was generated by digesting pHMW8-4 with *Spe*I, which cuts at a unique site within the *hia* gene, blunt-ending the resulting overhangs, and inserting a kanamycin-resistance cassette. Plasmid pHMW8-7 was generated by digesting pHMW8-3 with *Nru*I and *Hin*dIII, isolating the 7.3 kbp fragment (which contains pT7-7), blunt-ending and religating. (An additional *Hin*dIII site not shown in Fig. 2 is present adjacent to the right-most *Xba*I site of pHMW8-3; this site is derived from the pT7-7 polylinker region.)

Construction of H. influenzae *mutants*

In order to construct isogenic *H. influenzae* mutants, bacteria were made competent using the MIV method (Herriott *et al.*, 1970), and were transformed with linearized pHMW8-6, selecting for kanamycin resistance. Allelic exchange was confirmed by Southern analysis.

Analytical techniques

To identify recombinant proteins produced by *E. coli* lysates containing recombinant phage, representative lysates were solubilized in electrophoresis sample buffer, subjected to SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose (Barenkamp and Leininger, 1992). The nitrocellulose was then probed sequentially with the convalescent serum of the child infected with strain 11, and with alkaline phosphatase-conjugated goat anti-human IgG second antibody (Tago). To identify the native Hia proteins produced by non-typable *Haemophilus* strain 11, bacterial cell sonicates were subjected to gel electrophoresis and transfer steps as outlined above. The nitrocellulose sheets were then probed with rabbit antiserum raised against an Hia recombinant protein followed by reaction with an alkaline-phosphatase-conjugated goat anti-rabbit second antibody (Bio-Rad).

In order to examine plasmid-encoded proteins, the bacteriophage T7 expression vector pT7-7 was employed, and the relevant pT7-7 derivatives were transformed into *E. coli* BL21(DE3). Activation of the T7 promoter was achieved by inducing expression of T7 RNA polymerase with isopropyl-β-D-thiogalactopyranoside (final concentration, 1 mM). After induction for 30 min at 37°C, rifampicin was added to a final concentration of 200 μg ml^{-1}. Thirty minutes later, 1 ml of culture was pulsed with 50 μCi of *trans*-^{35}S label (ICN) for 5 min. Bacteria were harvested and whole cell lysates were resuspended in Laemmli buffer for analysis by SDS–polyacrylamide electrophoresis on 7.5% acrylamide gels. Autoradiography was performed with Kodak XAR-5 film.

DNA sequence analysis

DNA sequence analysis was performed by the dideoxy chain-termination method with the US Biochemicals Sequenase kit as suggested by the manufacturer. Several 21-mer oligonucleotide primers were generated as necessary to complete the sequence, and [^{35}S]-dATP was purchased from New England Nuclear. Data were analysed with CompuGene software (Barnes, 1987) and the Genetics Computer Group program from the University of Wisconsin (Devereux *et al.*, 1984) on a Digital VAX 8530 computer.

Adherence assays

Adherence assays were performed with Chang epithelial cells (Wong–Kilbourne derivative, clone 1-5c-4 (human conjunctiva)), which were seeded into 24-well tissue culture plates, as described (St. Geme *et al.*, 1993). Bacteria were inoculated into broth and allowed to grow to a density of approximately 2×10^9 colony-forming units (cfu) per ml. Approximately 2×10^7 cfu were inoculated onto epithelial cell monolayers, and plates were gently centrifuged at $165 \times g$ for 5 min to facilitate contact between bacteria and the epithelial surface. After incubation for 30 min at 37°C in 5% CO_2, monolayers were rinsed five times with phosphate-buffered saline (PBS) to remove non-adherent organisms and were treated with trypsin–EDTA (0.05% trypsin/0.5% EDTA) in PBS to release them from the plastic support. Well contents were agitated, and dilutions were plated onto solid medium to yield the number of adherent bacteria per monolayer. Percent adherence was calculated by dividing the number of adherent cfu per monolayer by the number of inoculated cfu. Quantitative results were confirmed by light microscopy performed as previously described (St. Geme and Falkow, 1990).

Southern analysis of genomic DNA

Chromosomal DNA was digested to completion with *Eco*RI and electrophoresed on 0.7% agarose–Tris acetate gels at 100 V. The DNA was transferred to Hybond-N^{3C} (Amersham) filters by capillary flow. Nick-translation labelling of the DNA probe and Southern hybridization were performed using standard techniques (Maniatis *et al.*, 1982; Silhavy *et al.*, 1984). Autoradiographs were prepared using Kodak XAR-5 film (Eastman Kodak Co.) and an intensifying screen.

Nucleotide sequence accession number

The sequence of the *hia* gene has been submitted to GenBank and assigned the accession number U38617.

Acknowledgements

We thank Linda Winter and David Cutter for expert technical assistance. This work was supported by Public Health Service Grant AI-21707 from the National Institute of Allergy and Infectious Diseases to S.J.B., and by an American Heart Association Grant-in-Aid, and an Infectious Diseases Society of America Young Investigator Award to J.W.S.

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